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Intrafollicular melatonin concentration is elevated in patients with ovarian hyperstimulation syndrome (OHSS) and can serve as an important predictor of OHSS

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Abstract

Purpose Melatonin is an important factor in regulating numerous processes in human female reproduction. The aim of the present study was to compare melatonin levels in the follicular fluid (FF) of ovarian hyperstimulation syndrome (OHSS) women with those of non-OHSS women undergoing in vitro fertilization (IVF)-embryo transfer and to evaluate the relationship between FF melatonin levels and IVF outcomes in these women.

Methods We determined FF melatonin levels in 20 OHSS women and 23 non-OHSS women on oocyte retrieval day.

Results OHSS patients had significantly higher melatonin levels as compared to the non-OHSS women ($P < 0.001$). In addition, melatonin levels of the patients were significantly positively correlated with antral follicle count (AFC), serum anti-Müllerian hormone (AMH) levels, serum estradiol (E_2) levels on human chorionic gonadotropin (HCG) administration day, number of retrieved oocytes, total fertilized oocytes, normally fertilized oocytes, cleaved zygotes, top quality embryos on day 3, blastocysts obtained and embryos suitable for transplantation (day 3 embryos + day 5/6 blastocysts) ($P < 0.05$). While, the intrafollicular melatonin levels were significantly negatively correlated with age, basal serum follicle-stimulating hormone (FSH) levels, serum FSH levels on HCG administration day ($P < 0.01$). Since younger women with more AFC, higher AMH levels, higher serum E_2 levels and larger number of retrieved oocytes are much easier to encounter OHSS, while FF melatonin levels are significantly correlated with these five indices in our study, we propose that intrafollicular melatonin concentration can also be an important predictor of OHSS.

Conclusions This is the first demonstration that FF melatonin levels were significantly higher in OHSS patients than in non-OHSS group and FF melatonin levels may serve as an important predictor of OHSS.

Keywords Melatonin · OHSS · AMH · IVF · Follicular fluid

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a common iatrogenic complication in patients undergoing in vitro fertilization (IVF) after gonadotropin stimulation, followed by human chorionic gonadotropin HCG) administration. The incidence of moderate and severe OHSS may occur in 3–10% of all assisted reproductive technique cycles and it may reach 20% among high-risk women [1, 2]. In fact, the real incidence of OHSS is probably much higher since the symptoms of the mild form of OHSS are not typical and easy to be ignored or misdiagnosed. Although the moderate clinical manifestations of OHSS are mainly some digestive symptoms abdominal discomfort, nausea, vomiting, diarrhea), it can be a potentially life-threatening condition in its severe forms which include hepatorenal failure, acute

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respiratory distress syndrome, hemorrhage from ovarian rupture, thromboembolism and even death, resulting in hospitalization in 1.9% of case [3, 4]. Some severe OHSS may even result in death.

OHSS increases the physical, psychological and economic burden of the patients. There is no method that can completely abolish OHSS and the prevention of OHSS is based on its prediction. Since the prevention of OHSS can be lifesaving and is principally preferred over its treatment, the prediction of OHSS becomes extremely important. The primary risk factors for OHSS are young age, polycystic ovarian syndrome (PCOS), the history of previous OHSS, etc. [5]. However, none is capable of independently forecasting OHSS. To date, the prediction and prevention of OHSS still remain a question for clinicians.

Although the pathogenesis of this state is not completely understood, ovarian response during controlled ovarian stimulation is directly correlated with the risk of OHSS. Excessively high estradiol (E_2) levels produced by the ovarian follicles, in the presence of human chorionic gonadotropin (HCG), sometimes may cause the excessive production of the vascular endothelial growth factor (VEGF) and some inflammatory factors (interleukins, tumor necrosis factor, etc.) [6–11]. The overabundance of VEGF and inflammatory factors causes the dilatation of vascular endothelium which leads to a massive shift of the body fluids from the vessels into the third space. Therefore, the serum E_2 levels on HCG day with/without the number of the follicles on HCG day can be used as a predictive index of OHSS [12].

Melatonin is an indoleamine produced mainly by the pineal gland which can be regulated by the circadian rhythms [13, 14]. The importance of melatonin involved in female reproduction has been the focus for research in recent years, although there are controversial opinions about the effects of melatonin on female reproductive axis [15, 16]. Higher levels of melatonin are found in human follicular fluid FF) than in plasma because melatonin is not only derived from the general circulation but also synthesized in the ovary [17–21]. Furthermore, since melatonin and its metabolites are free radical scavengers, more attention has been paid to the beneficial impact of melatonin as a supplement in the culture medium of oocytes and embryos.

In OHSS patients, high levels of active free radicals are produced not only by the standard IVF procedures such as superovulation itself but also by the overabundance of VEGF and inflammatory factors which are produced during the pathophysiological process of OHSS [22]. To date, little is known about the intrafollicular melatonin levels OHSS patients, and a focus on its association with IVF outcomes also needs to be investigated. We designed this study to determine the intrafollicular melatonin levels in OHSS patients and non-OHSS women undergoing IVF. Furthermore, we also evaluated the relevance of melatonin concentration to clinical parameters of OHSS and IVF outcomes.

Materials and methods

Participants

43 females (20–35 years old) scheduled for IVF from April 2016 to May 2016 were enrolled into this research in the Center for Reproductive Medicine, Renji Hospital, School of Medicine, Shanghai Jiao Tong University. Approval for this study was obtained from the Ethics Committee of Renji Hospital. Informed consent was signed by each patient recruited into this study.

Inclusion criteria of OHSS group were free from major medical illness, with manifestation of clinical symptoms (nausea, vomiting, diarrhea, abdominal distension, abdominal pain, oliguria, breathing difficulties, etc.), with or without enlargement of ovaries ($\geq$ 5 cm), ascites, hydrothorax in 14 days after oocyte retrieval [23]. For group non-OHSS, the inclusion criteria were with neither clinical symptoms (nausea, vomiting, abdominal distension) nor enlargement of ovaries ($\geq$ 5 cm) in 14 days of the oocytes retrieval. Exclusion criteria were personal history of hyperthyroidism, hypothyroidism, diabetes mellitus, tuberculosis, heart diseases, hypertension, endometriosis and relevant systemic diseases. Patients with chronic use of any medication and digestive diseases were also excluded.

Assisted reproduction procedures and follicular fluid sampling

Venous blood of the patients was taken at 8 a.m. on the second or third day of their menstrual cycle after overnight fasting using a vacutainer system. Blood samples were used to determine basal serum follicle-stimulating hormone (bFSH), luteinizing hormone (bLH), E_2, testosterone (T), prolactin (PRL), thyrotropin (TSH), AMH, glucose levels and other related indices by chemiluminescence immunoassay or enzyme-linked immunosorbent assay in our center's reproductive endocrinology laboratory. The corresponding demographic and related medical data of the patients were collected on the same day.

The clinicians chose the proper IVF procedures (long agonist protocol/short agonist protocol/antagonist protocol/minimal stimulation protocol) for these patients based on patients' ovarian reserve and clinicians' experience. The initial gonadotropin (Gn) dose was individually determined depending on the patients' age, Body Mass Index (BMI), ovarian reserve and ovarian response in previous cycles. The ovary and follicle development was supervised by intravaginal ultrasound examination; the serum sex hormones were

determined every two or three days until the day of HCG application. Gonadotropin doses were adjusted based on the patients' response to ovarian stimulation, serum hormone levels and the quantity and size of the ovarian follicles. When the leading follicle reached 20 mm in meandiameter with a serum E_2 level of 200–300 pg/ml per mature follicle (≥ 14 mm), HCG or gonadotropin-releasing hormone agonist (GnRHa) was injected as the trigger.

Ultrasound-guided transvaginal follicular aspiration was performed after 34–36 h of the HCG/GnRHa trigger injection. The dominant FF (diameter 19–24 mm) without any blood contamination from each patient was immediately collected and stored on ice. All follicular aspirations were performed between 8 and 11 a.m. The FF samples were then centrifuged at 1000 RCF at 0 °C for 20 min immediately. The supernatants were collected and stored at − 20 °C until further analysis within two months. All procedures were performed under protection from light.

Determination of melatonin in the FF

The melatonin direct radioimmunoassay kit (DIAsource ImmunoAssays S.A. Belgium) was used to determine FF melatonin concentrations. The assays were performed as per the manufacturer's instructions. The inter-assay and intra-assay coefficients of variation were 9.75% and 9.5%, respectively. The minimum detectable melatonin level with this kit was 2.3–1000 pg/ml.

Statistical analysis

All calculations were performed by the Statistical Package for Social Science (version 19.0; SPSS, Inc, Chicago, IL, USA). Normally distributed continuous variables were presented as mean ± standard deviation (SD). Variables with a highly skewed distribution were presented as medians (interquartile range).

Independent samples *T* test for the normally distributed continuous variables and Mann–Whitney *U* test for the non-normally distributed continuous variables were used to compare the differences between the two groups. The simple linear regression and the Spearman's simple correction were used to check the associations between two variables. The confidence interval was set at 95% for all tests and $P < 0.05$ was considered as significantly different.

Results

Intrafollicular levels of melatonin and baseline parameters of patients in two groups

Baseline parameters and intrafollicular concentrations of melatonin of the patients allocated to the subgroups are described in Table 1. Women in OHSS group showed significantly higher levels of FF melatonin compared to non-OHSS group ($P < 0.001$). In addition, as expected, women in OHSS group showed significantly higher serum levels of AMH, higher AFC and lower basal serum FSH levels compared to non-OHSS group ($P < 0.01$). No significant difference in age, duration of infertility, menstrual cycle, bLH, bE_2, T, TSH, BMI was observed between OHSS group and non-OHSS group.

Comparisons of IVF outcomes of patients in two groups

The IVF outcomes of patients in the subgroups are shown in Table 2. For women in OHSS group, significantly higher numbers of retrieved oocytes, total fertilized oocytes, normal

Table 1 Baseline parameters of patients and intrafollicular melatonin levels on the day of oocyte retrieval

Parameters	Group OHSS ($n = 20$)	Group non-OHSS ($n = 23$)	*P* value
Age (years)	29.45 ± 4.48	30.83 ± 3.57	0.326
Body Mass Index (kg/m^2)	21.88 (19.70–26.63)	20.35 (18.78–22.90)	0.128
Menstrual cycle (days)	31.28 ± 5.48	29.38 ± 4.57	0.228
Infertility duration (years)	3.00 (2.00–5.75)	3.00 (2.00–6.00)	0.970
Antral follicle count (n.)	20.50 (16.25–27.00)	10.00 (6.75–13.00)	< 0.001
Intrafollicular melatonin (pg/ml)	44.80 (27.53–179.00)	11.58 (0.26–25.74)	< 0.001
Serum AMH (ng/ml)	10.95 ± 4.93	3.23 ± 1.28	< 0.001
Basal serum FSH (IU/ml)	6.03 (4.86–6.60)	7.15 (6.11–8.91)	0.001
Basal serum LH (IU/ml)	6.14 (4.10–8.33)	4.27 (3.58–7.56)	0.165
Basal serum E_2 (pg/ml)	33.82 ± 14.85	44.05 ± 18.46	0.054
Serum T (ng/dl)	1.07 ± 0.70	0.81 ± 0.43	0.152
Serum TSH (IU/ml)	2.07 (1.52–4.09)	2.24 (1.56–3.52)	0.670

Table 2 Comparisonsof IVF outcomes of the OHSS and non-OHSS patients

Parameters	Group OHSS ($n = 20$)	Group non-OHSS ($n = 23$)	P value
Duration of stimulation (days)	9.00 (8.00–10.75)	9.00 (8.00–10.50)	0.881
Total dose of FSH (IU)	1325.00 (981.25–1525.00)	1275.00 (412.50–1518.75)	0.312
Initial dose of FSH (IU)	150.00 (125.00–150.00)	150.00 (140.63–175.00)	0.876
Total dose of HMG (IU)	0.00 (0.00–0.00)	0.00 (0.00–412.50)	0.008
Serum FSH on day HCG (IU/ml)	9.01 (7.99–10.81)	12.08 (9.66–14.90)	0.002
Serum LH on day HCG (IU/ml)	2.38 (1.43–5.05)	3.84 (1.58–6.06)	0.158
Serum E_2 on day HCG (pg/ml)	7915.85 ± 1585.81	2660.26 ± 1842.23	< 0.001
Retrieved oocytes (n.)	25.00 (22.00–33.25)	9.00 (6.00–12.25)	< 0.001
Total fertilized oocytes (n.)	19.00 (15.00–24.75)	7.00 (3.75–10.00)	< 0.001
Normal fertilized oocytes (n.)	16.50 (13.00–19.75)	5.50 (3.75–7.25)	< 0.001
Cleaved zygotes (n.)	16.00 (13.00–20.75)	6.00 (3.75–7.25)	< 0.001
Top quality embryos on day 3 (n.)	6.00 (5.00–13.50)	3.00 (1.00–4.25)	< 0.001
Obtained blastocysts (n.)	4.50 (2.00–6.00)	2.00 (0.00–3.00)	0.001
Top quality blastocysts (n.)	0.00 (0.00–1.00)	0.00 (0.00–1.25)	0.935
Total embryos suitable for transplantation (n.)	7.50 (5.25–9.00)	4.00 (1.00–6.00)	< 0.001

Embryo quality was determined according to Peter's integrated morphology cleavage embryo score and Garnder's blastocyst quality score, top quality embryos on day 3 is defined as 6–10 blastomeres on the 3rd day of the fertilization with complete pellucid zones and the degree of the fragmentation ≤ 10%. Top quality blastocyst is defined as the fully expanded blastocysts with blastocoel completely filling the embryo, the blastocyst hatches partially or totally out of the pellucid zone; tightly packed, many inner cells or loosely grouped, several inner cells; many trophectodermcells forming a cohesive epithelium or a few trophectoderm cells forming a loose epithelium [24, 25]. Stimulation days were defined as the days on which FSH, LH, HMG, Clomifene Citratewere given to patients based on their ovarian stimulation protocol (not concluding HCG day)

fertilized oocytes, cleaved zygotes, top quality embryos on day 3, blastocysts obtained and embryos suitable for transplantation (day 3 embryos + day 5/6 blastocysts)were observed when compared to women in non-OHSS group ($P < 0.05$).

Intrafollicular melatonin levels are significantly correlated with the ovarian reserve markers and are highly relevant markers of OHSS

The intrafollicular levels of melatonin were significantly negatively correlated with age (Fig. 1a) and bFSH (Table 3), and were significantly positively correlated with AFC (Fig. 1b), serum AMH levels (Fig. 1c). No significant correlation was observed between FF melatonin levels and BMI, the duration of infertility, bLH levels, bE_2 levels, T levels and TSH levels. To evaluate the correlation of high predictive indexes of OHSS to the intrafollicular melatonin levels, correlations of intrafollicular melatonin levels and the E_2 levels on HCG day, number of retrieved oocytes were also analyzed. The results demonstrated that FF melatonin levels were significantly positively correlated with the E_2 levels on HCG day (Fig. 1d) and the number of retrieved oocytes (Fig. 1e). No significant correlation was observed between FF melatonin levels and serum LH levels, serum P levels on HCG administration day as well as total FSH dose, starting FSH dose, total HMG dose, days of the Gn received.

Relationship between FF melatonin levels on the day of oocyte retrieval and IVF outcomes

The results demonstrated that melatonin levels were significantly positively correlated with the number of retrieved oocytes, total fertilized oocytes, normally fertilized oocytes, cleaved zygotes, top quality embryos on day 3, blastocysts obtained and embryos suitable for transplantation (day 3 embryos + day 5/6 blastocysts) ($P < 0.05$) (Table 4), whereas no significant correlation was observed between FF melatonin levels and number of top-quality blastocysts on day 5/6.

Discussion

In this study, we determined the intrafollicular melatonin levels in OHSS and non-OHSS women undergoing IVF and investigated their relationships with clinical parameters and IVF outcomes. We found that OHSS women showed significantly higher levels of FF melatonin as compared to non-OHSS women. Higher intrafollicular levels of melatonin were associated with younger age, higher AFC, higher serum AMH levels, higher serum E_2 levels on HCG day, lower serum basal FSH levels and better IVF outcomes.

At present, the treatments for OHSS are mostly pointing to the symptoms and there are few effective etiological

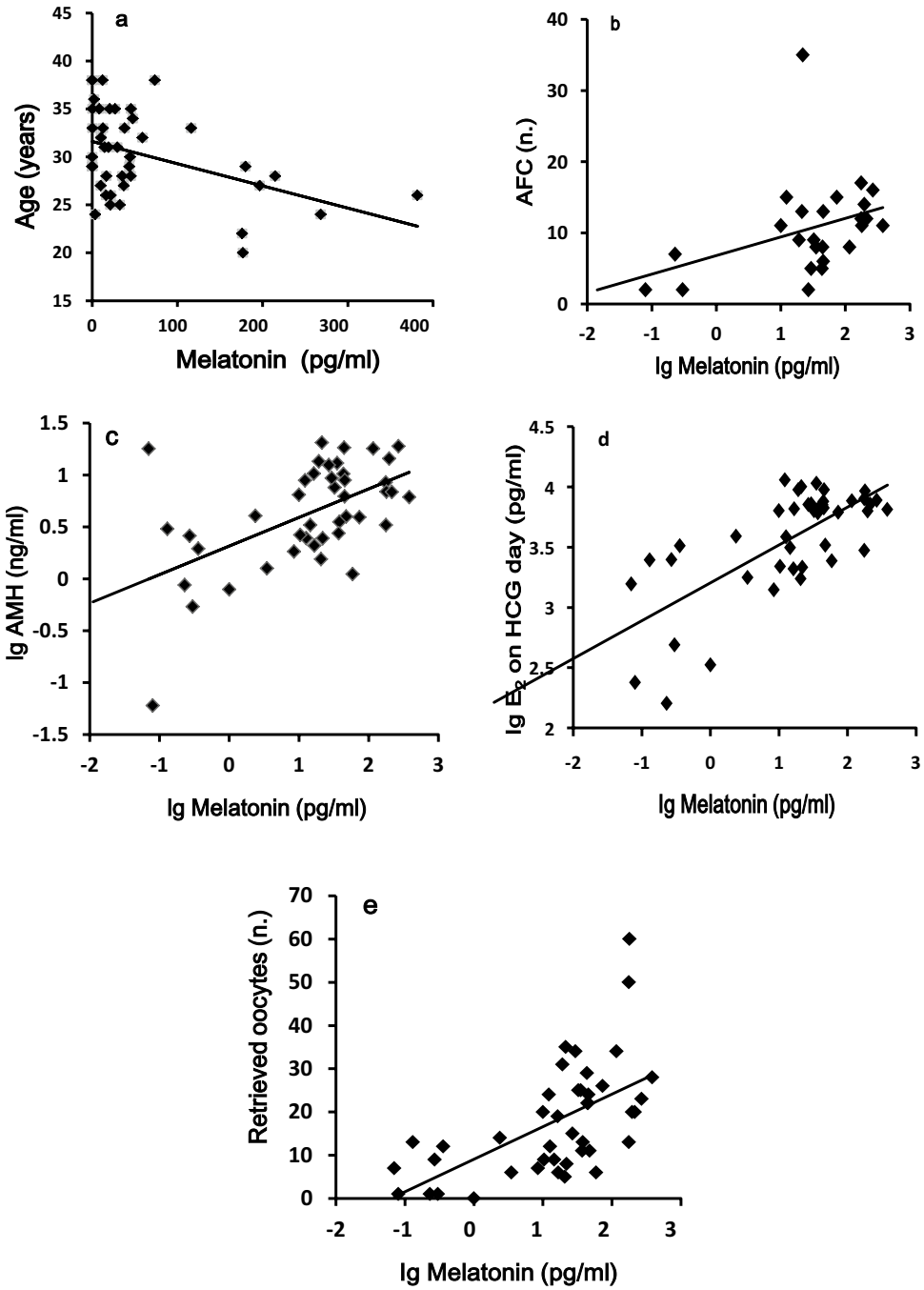

Fig. 1 Correlation between the FF melatonin levels on the oocyte retrieval day and age, AFC, serum AMH levels, serum E_2 levels on HCG day and number of retrieved oocytes. **a** The intrafollicular levels of melatonin are negatively correlated with age (Spearman–Rho $r_s = -0.387$, $P = 0.010$); **b** the FF melatonin levels are positively correlated with AFC (Spearman–Rho $r_s = 0.484$, $P = 0.001$), **c** the FF melatonin levels are positively correlated with serum AMH levels (Spearman–Rho $r_s = 0.478$, $P = 0.001$), **d** the FF melatonin levels are positively correlated with the E_2 levels on day HCG (Spearman–Rho $r_s = 0.598$, $P < 0.001$), **e** the FF melatonin levels are positively correlated with the number of retrieved oocytes (Spearman–Rho $r_s = 0.614$, $P < 0.001$)

treatments for this syndrome; prevention of OHSS remains a crucial problem for clinicians. Identifying the OHSS risk factors could not only be useful to better understanding the pathophysiology of this disease, but also be helpful for taking precautions to reduce the incidence. Therefore, the need to identify high-risk OHSS patients is imperative. The predictive factors of OHSS include young age, low BMI, higher serum AMH levels, PCOS, high-dose gonadotropin treatment, high serum E_2 levels and large number of oocytes ≥ 14 mm on HCG day, HCG administration as trigger, larger number of retrieved oocytes and pregnancy [26]. But to date, none of these parameters has been shown as an independent predictor of OHSS. In our research, intrafollicular melatonin levels are significantly correlated with age, AFC, serum AMH levels, serum E_2 levels on HCG day and number of retrieved oocytes; therefore, we propose that intrafollicular melatonin levels can act as a good predictor for OHSS.

Prior study has demonstrated that ovarian-produced reactive oxygen species (ROS) plays an important role as second messengers in the expression of genes related

Table 3 Correlation between intrafollicular melatonin levels and baseline parameters, ovarian stimulation parameters in subgroups

Parameters	Intrafollicular melatonin levels (pg/ml) Rank correlation coefficient of spearman	*P* value
BMI (kg/m^2)	0.017	0.916
Basal serum FSH (IU/L)	− 0.357	0.019
Basal serum LH (IU/L)	− 0.051	0.746
Basal serum E_2(pg/ml)	− 0.165	0.289
Duration of stimulation (days)	0.075	0.631
Total dose of FSH (IU)	0.272	0.077
Initial dose of FSH (IU)	0.144	0.357
Serum FSH on day HCG (IU/L)	− 0.437	0.003
Serum LH on day HCG (IU/L)	− 0.233	0.132
Serum P on day HCG (IU/L)	0.228	0.141

Table 4 Correlation between intrafollicular melatonin levels and IVF outcomes of the patients

Parameters	Intrafollicular melatonin levels (pg/ml) Rank correlation coefficient of spearman	*P* value
Fertilized oocytes (n.)	0.518	< 0.001
Normal fertilized oocytes (n.)	0.506	0.001
Cleaved zygotes (n.)	0.507	0.001
Top quality embryos on day 3 (n.)	0.418	0.005
Blastocysts obtained (n.)	0.360	0.018
Top quality blastocysts (n.)	0.170	0.277
Embryos suitable for transplantation (n.)	0.351	0.021

to oocyte maturation [27]. The major source of ROS in the ovary appears to be macrophages, neutrophils and steroidogenically active cells such as GCs of antral follicles, which generate tremendous numbers of free radicals [28–30]. These radicals not only act in the regulation of ovulation, but also induce apoptosis of ovarian cells through deteriorating cell membrane lipids and destroying DNA [31, 32]. Therefore, the number of free radicals must be carefully regulated to maintain homeostasis. If these free radicals are above the acceptable limits and break the balance between the antioxidants and apoptosis, molecular damage is induced in the oocytes, which eventually has negative consequences. Elevated melatonin in follicles is likely to protect the oocytes from the damage caused by free radicals.Those follicles that cannot survive the free radicals' damage and cannot be chosen by the circulating levels of FSH would die of atresia [21].

Our study demonstrated that the FF melatonin levels are significantly and positively correlated with the number of the retrieved oocytes and the levels of AMH which reflects the state of ovarian reserve. This may also be due to the melatonin's effective protective function of the primordial, preantral and antral follicles from being deteriorated by the free radicals; so, the oocytes can survive in the microenvironment filled with FF under ovarian hyperstimulation. These protective pathways might be: (a) melatonin binds to the melatonin receptors which are located on the plasma membrane and the nuclear membrane and boosts the transcription of a number of genes (superoxide dismutase, glutathione peroxidase, catalase) that can be served as anti-free radical scavengers or inhibit the transcription of some genes (nitric oxide, etc.) acting as free radicals [33]. (b) It also binds to the free radicals directly due to its lipophilic nature and generates a series of metabolites which are also effective free radical scavengers [34]. Through these two pathways, melatonin protects the quantity and quality of oocytes from being damaged by the free radicals. When the oocytes become mature under the stimulation of gonadotropins, the large amount of E_2 produced during this process leads to the production of VEGF and other associated cytokines with the collaboration of HCG which induces the dilation of vascular endothelium and subsequently OHSS occurs.

FF melatonin can protect not only the oocytes but also protect the GCs in the FF from being deteriorated by free radicals. GCs play an important role in establishing an essential microenvironment for the oocytes. One of the OHSS predictors AMH is produced by the GCs of preantral follicles and small antral follicles [35]. The higher FF melatonin levels in OHSS patients protect the GCs better from being damaged by the ROS, thus the GCs in OHSS patients produce more AMH than non-OHSS women. So as we have shown in our study, FF melatonin levels were positively correlated with AMH levels. In our study, the FF melatonin levels were also significantly negatively correlated with age. The same tendency was also found by other researchers in serum and urinary melatonin levels with age [36, 37]. We speculate that with increasing age, the remaining primordial, preantral follicles in the ovarian reserve pool becomes less and less; subsequently, the number of GCs where melatonin is produced in the ovary also becomes fewer. Therefore, the FF melatonin levels decrease as age increases.

The antioxidative and antiapoptotic properties of melatonin seem to produce positive effects in treating many diseases [38–41]. The efficiency of oral melatonin administration in infertile women with poor ovarian reserve has been validated by clinical trials [42]. Since the acute and chronic toxicity of melatonin is extremely low, the oral dosage of melatonin can even be one gram per day. Therefore,

remind of augmentation of risk of OHSS should be given to women with better ovarian reserve when melatonin is given to women undergoing IVF, and more attention should be given to prevent OHSS in these patients.

In conclusion, we show for the first time that FF melatonin levels are higher in OHSS patients than non-OHSS group and FF melatonin concentration can be a reliable predictor of OHSS. However, large-scale, prospective studies are needed to validate our result. In addition, in our study, all of the patients are mild or moderate OHSS; thus, FF melatonin levels in severe OHSS patients should also be investigated further.

Author contributions CZ: project development and editing, MZ: tissue collection, data analysis, manuscript writing and project development, GZ: tissue collection, JT: tissue collection, W-PL: project development, Z-JC: project development.

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Compliance with ethical standards

Conflict of interest All of the authors declare that they have no competing interests.

Ethical approval All procedures performed in this study involving were in accordance with the ethical standards of the institutional research committee (no. 2015030308) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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